QP501.J6

STIC-ILL

Fr m: Steadman, David (AU1652)
S nt: Tuesday, August 20, 2002 1:10 PM
To: STIC-ILL
Subject: literature request for 09/815,533

Art Unit: 1652
Office: 10D-04
Mailbox: 10C-01 M3
Case Serial #:09/815,533

Please provide the following references:

1) J Chromatogr 1990 Feb 23;525(2):297-306
Purification of urokinase by combined cation exchanger and affinity chromatographic cartridges.
Hou KC, Zaniewski R.

2) Purification of high-molecular-weight and low-molecular-weight urokinase and kinetic study
Sun, Tian-Xiao; Wang, Hong-Mei; Xu, Chang-Fa
Shengwu Huaxue Zazhi (1997), 13(3), 344-349

3) Isolation, purification and comparative studies of certain properties of high- and low-molecular-weight urokinases of human urine.
Sun, Leqin; Zhang, Hongzu; Zhu, Dexu
Shengwu Huaxue Yu Shengwu Wuli Xuebao (1984), 16(3), 303-6

4) J Biochem (Tokyo) 1981 Jul;90(1):225-32
A comparative study of high molecular weight urokinase and low molecular weight urokinase.
Nobuhara M, Sakamaki M, Ohnishi H, Suzuki Y.

5) Enzyme 1981;26(4):221-4
Kinetic studies of three different molecular forms of urokinase for the activation of native human plasminogen.
Toki N, Takasugi S, Sumi H.

6) Thromb Haemost 1983 Apr 28;49(2):91-5
Purification of high molecular weight urokinase from human urine and comparative study of two active forms of urokinase.
Shibatani T, Kakimoto T, Chibata I.

7) Thromb Haemost 1982 Jun 28;47(3):197-202
Rapid isolation of high molecular weight urokinase from native human urine.
Huber K, Kirchheimer J, Binder BR.

8) Chem Pharm Bull (Tokyo) 1981 Feb;29(2):463-71
Comparative studies on two active enzyme forms of human urinary urokinase. I. Purification by serial column chromatography and homogeneity analyses of molecular weight and isoelectric point.
Miwa N, Takayanagi H, Suzuki A.

Thank you,
David J. Steadman
Art Unit 1652
CM1, 10D-04
308-3934

J. Biochem. 90, 225-232 (1981)

A Comparative Study of High Molecular Weight Urokinase and Low Molecular Weight Urokinase

Masahiro NOBUHARA, Michio SAKAMAKI, Haruo OHNISHI, and Yasuo SUZUKI

Tokyo Research Laboratory, Mochida Pharmaceutical Co., Ltd., Kita-ku, Tokyo 113

Received for publication, January 13, 1981

Two forms of urokinase [EC 3.4.99.26] with molecular weights of 51,600 and 34,500 were purified from human urine. The specific activities of the high molecular weight urokinase (HMW-UK) and low molecular weight urokinase (LMW-UK) were 157,400 and 246,700 International Units (IU/mg), respectively. Purified HMW-UK was 97% active and LMW-UK was 88% active, as judged by using *p*-nitrophenyl-*p'*-guanidinobenzoate. LMW-UK had five multiple isoelectric subforms, compared with HMW-UK which had only one. Not only HMW-UK but also LMW-UK was composed of two polypeptide chains linked by disulfide bond(s). The molecular weight of the heavy chain of both forms was the same (34,000 daltons), while the molecular weight of the light chain of HMW-UK was 17,600 and that of LMW-UK was approximately 1,200–3,400. Enzyme kinetic studies revealed that the kinetic constants, K_m and k_{cat}, of both forms toward the synthetic substrates, acetyl-Gly-Lys-methylester (AGLMe) and glutaryl-Gly-Arg-4-methylcoumarin-7-amide (GGA-MCA), were almost the same, but the dissociation constant of HMW-UK toward Glu-plasminogen was 2.4–2.6 times less than that of LMW-UK. HMW-UK incubated at 37°C was converted into LMW-UK in an autocatalytic digestion manner leading to no loss of the total activity. These results show that HMW-UK with a higher affinity toward Glu-plasminogen is converted into LMW-UK with a lower affinity, a greater portion of the light chain of HMW-UK splitting off.

Urokinase, a plasminogen activator, has been purified from human urine and characterized (*1–3*). Recently two molecular forms of urokinase with molecular weights of 47,000–55,000 and 31,000–34,000 have been isolated and then it became clear that HMW-UK is composed of two

Abbreviations: HMW-UK, high molecular weight urokinase; LMW-UK, low molecular weight urokinase; SDS, sodium dodecyl sulfate; AGLMe, acetyl-Gly-Lys-methylester; GGA-MCA, glutaryl-Gly-Arg-4-methylcoumarin-7-amide; VLL-pNA, H-D-Val-Leu-Lys-*p*-nitroanilide; K_{plg}, dissociation constant for activation of Glu-plasminogen; k_{plg}, catalytic rate constant for activation of Glu-plasminogen.

chains linked by disulfide bond(s) and the heavy chain has the active site serine (*4, 5*). It is, however, not obvious 1) whether LMW-UK also has two chains, 2) whether LMW-UK is derived from HMW-UK, and 3) which form of urokinase activates effectively Glu-plasminogen for plasmin. Thus we have attempted to isolate the two molecular forms of urokinase in highly purified states in order to clarify these points.

EXPERIMENTAL PROCEDURE

Purification of Urokinase—The crude urokinase, which was prepared by use of polyacrylonitrile fiber column chromatography and ammonium sulfate precipitation (*6*), was dissolved in 0.01 M phosphate buffer (pH 6.0) and dialyzed overnight against the same buffer. The urokinase solution was passed through a column of Amberlite CG-50 (Rohm and Hoas Co.) equilibrated with 0.01 M phosphate buffer (pH 6.0). The column was subsequently washed with 0.05 M phosphate buffer (pH 7.0) until no absorbance at 280 nm was detected, and urokinase was eluted by changing the buffer to 0.01 M phosphate buffer (pH 8.0) containing 0.5 M NaCl, followed by gel filtration on a 10×91 cm column of Sephadex G-100 (Pharmacia Fine Chemicals) with 0.1 M phosphate buffer (pH 8.0) containing 0.3 M NaCl. Pooled fractions corresponding to HMW-UK and LMW-UK were dialyzed overnight against 0.1 M phosphate buffer (pH 7.0) containing 0.4 M NaCl, and subsequently chromatographed on a 5×20 cm column of Sepharose-ε-aminocaproylbenzamidine equilibrated with the same buffer (*7*). Urokinase was then eluted with 0.1 M acetate buffer (pH 4.0) containing 0.4 M NaCl and gel filtration on a column of Sephadex G-100 was carried out. HMW-UK and LMW-UK thus purified were lyophilized. All procedures were performed at 4–10°C.

Electrophoresis—Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed at a gel concentration of 7.5% or 15% and protein was stained with 0.25% Coomassie brilliant blue in 7% acetic acid with 50% ethanol (*8*). Gels were scanned with a Cosmo densitometer (model ACE D109) at 500 nm.

Isoelectric focusing with 1% Ampholine in the pH range of 3.0–10.0 was performed in a 110 ml column (LKB) at 900 V for 72 h and the protein concentration of each fraction was determined by absorbance at 280 nm.

Molecular Weight Determination—The molecular weights of samples were determined by SDS gel electrophoresis and gel filtration on a 2.5×90 cm column of Sephadex G-100 equilibrated with 0.1 M phosphate buffer (pH 8.0) containing 0.3 M NaCl using the following proteins as references; bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,700), and cytochrome *c* (12,500).

Amino Acid Analysis and Carbohydrate Content—Protein samples (1.7–3.5 mg) were hydrolyzed with 1 ml of 6 N HCl with 0.2% phenol at 110°C for 22 and 72 h, and the amino acid compositions were analyzed with a HITACHI KL-5 amino acid analyzer (*9*). Tryptophan was determined by the method of Edelhoch (*10*). The sialic acid, amino sugar and neutral sugar contents of urokinase (0.8–1.1 mg) were analyzed by the methods of Warren (*11*), Elson and Morgan (*12*), and Anno and Seno (*13*), respectively.

Determination of Urokinase Activity—The fibrin plate method (*14*) was employed for the electrophoresis experiments, and the Two Step method (*15*) was adopted for the determination of the specific activity. The urokinase activity was expressed as IU using reference urokinase (MM-003, supplied by the National Institute of Hygienic Sciences, Tokyo).

Titration with *p*-nitrophenyl-*p*′-guanidinobenzoate (kindly provided by Dr. Iwanaga, Kyushu University) was performed at 25°C (*16*).

The amount of protein was determined by the method of Lowry *et al.* (*17*) with bovine serum albumin as standard. For titration with *p*-nitrophenyl-*p*′-guanidinobenzoate, the protein concentration of samples was estimated by absorbance at 280 nm using $A^{1\%}_{1\text{cm}} = 16.1$ for HMW-UK and 11.5 for LMW-UK in 0.1 N NaOH.

Kinetic Studies of Urokinase—Human Glu-plasminogen, human plasmin and H-D-Val-Leu-Lys-*p*-nitroanilide (VLL-pNA) were purchased from KABI, Sweden, and GGA-MCA and AGLMe were obtained from the Protein Research Foundation, Osaka.

a) GGA-MCA: GGA-MCA (1 ml, 0.12–1.43 mM) and urokinase (0.05 ml, 12.5 nM of HMW-UK, 14.5 nM of LMW-UK) were mixed at 37°C for 10 min in Tris-HCl buffer (pH 8.0)

containing 0.1 M NaCl. The reaction was stopped with 1.5 ml of 17% acetic acid and the amount of 7-amino-4-methylcoumarin released was determined (*18*).

b) AGLMe: AGLMe (0.5 ml, 0.25–5.0 mM) and urokinase (0.5 ml, 4.2 nM of HMW-UK, 4.8 nM of LMW-UK) were mixed, incubated at 37°C for 30 min in 0.06 M phosphate buffer (pH 7.5) containing 0.09 M NaCl, and then 0.5 ml of 0.75 M perchloric acid was added to the mixture. The amount of methanol released was determined (*19*).

c) Human Glu-plasminogen: The activation of plasminogen was estimated by a coupled assay with fibrinolysis (*15*) and VLL-pNA hydrolysis (*20*). In the case of fibrinolysis, Glu-plasminogen (0.1 ml, 390–2,340 μM) and urokinase (0.1 ml, 166.7 pM of HMW-UK, 193.2 pM of LMW-UK) were incubated at 37°C for 7 min, followed by addition of 0.7 ml of 0.07% fibrinogen and 0.1 ml of thrombin (10 u/ml). The time until the occurrence of fibrin clot lysis was measured. Hydrolysis of VLL-pNA was started by the mixing of Glu-plasminogen (0.2 ml, 156–1,560 μM), VLL-pNA (0.7 ml, 0.865 mM) and urokinase (0.1 ml, 16.7 pM of HMW-UK, 19.3 pM of LMW-UK) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.012 M NaCl. After 10 min incubation at 37°C the reaction was stopped with 0.1 ml of 5% acetic acid.

In every case urokinase was dissolved in 0.1% gelatin containing buffer. The catalytic rate constant and the dissociation constant for activation of Glu-plasminogen were designated as k_{plg} and K_{plg}, respectively.

Autocatalytic Conversion of HMW-UK to LMW-UK—HMW-UK (2.4 mg/ml) in 0.01 M phosphate buffer (pH 7.2) was incubated at 37°C with either 500 KIU/ml of aprotinin (Mochida Pharm. Co.) or 5 μM of benzamidine. Aliquots were taken at the indicated times in Fig. 4 and stored at −20°C until use. After SDS gel electrophoresis of each aliquot, the gels were either stained for the protein assay or cut into 1 mm slices for the activity determination which was done by incubation of the slices on a fibrin plate at 37°C for 7.5 h.

The caseinolytic activity of HMW-UK was examined by the following method in order to prove that the HMW-UK preparation was not contaminated with protease from urine (except plasminogen activating activity); HMW-UK (1 ml, 1.2 mg/ml) in 1/15 M phosphate buffer (pH 7.4) with 0.1% gelatin was incubated at 37°C for 60 min with 1 ml of 1% casein, prior to the addition of 10% trichloroacetic acid. The mixture was centrifuged at 3,000 rpm for 5 min and the absorbance at 280 nm of the supernatant was then measured.

Sialidase Treatment of LMW-UK—A mixture of 1.2 mg of LMW-UK and 0.2 u of sialidase (Sigam, Type VI from *Cholera perfringens*) in 1 ml of 1 M acetate buffer (pH 4.5) was incubated at 37°C for 2 h and subsequently subjected to isoelectric focusing (pH 3.0–10.0).

RESULTS

Purification of Urokinase—From the initial 25,000 liters of urine containing 175×10^6 IU of urokinase, 14×10^6 IU of HMW-UK and 11×10^6 IU of LMW-UK were obtained.

Molecular Weight and Other Properties of Urokinase—On isoelectric focusing HMW-UK showed a single peak at pH 9.70 although LMW-UK had five multiple isoelectric subforms ranged between pH 6.85 and 9.37. Sialidase treated LMW-UK also had five multiple subforms which had almost the same p*I*s as LMW-UKs' (Fig. 1). The molecular weights of HMW-UK and LMW-UK were determined to be 56,000 and 34,500 by SDS gel electrophoresis (Fig. 2), and 51,000 and 33,000 by gel filtration (Table I). The reduced HMW-UK gave two protein bands with molecular weights of 34,000 and 17,600. On the other hand, the reduced LMW-UK gave one band with a molecular weight of 34,000 which was practically identical with that of LMW-UK and the heavy chain of HMW-UK (Fig. 2). When 400 μg of the reduced LMW-UK per gel was submitted to SDS gel electrophoresis, a band with a molecular weight of 1,200–3,400 was apparently detected although the same amount of LMW-UK did not give any component in the same molecular weight range (Fig. 3). Several physicochemical and enzymological properties of HMW-UK and LMW-UK are summarized in Table I. Table II shows that neither HMW-UK nor LMW-UK contained sialic acid, although both urokinases had a small amount of neutral sugar (1.4–1.5%) and amino sugar (1.9–2.5%). The two forms of urokinase showed similar amino acid compositions, except

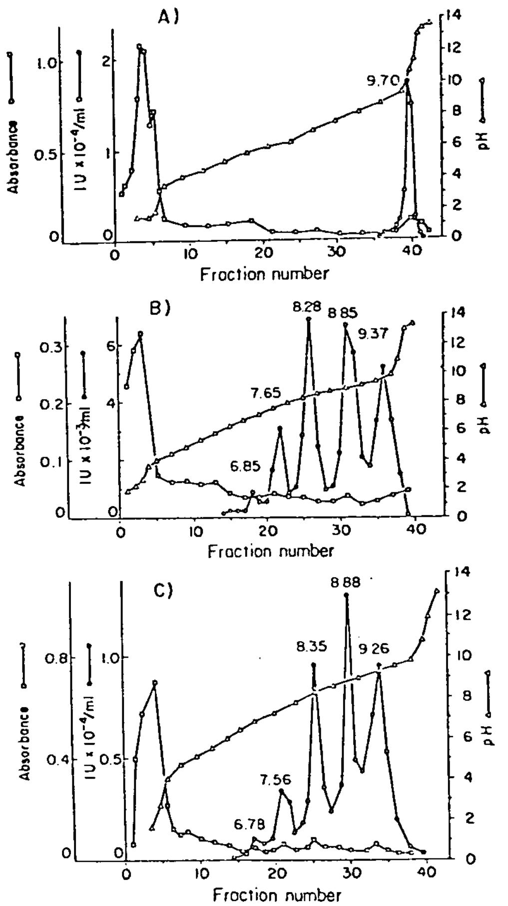

Fig. 1. Isoelectric focusing of HMW-UK, LMW-UK, and sialidase treated LMW-UK. HMW-UK (150,000 IU), LMW-UK (160,000 IU), and sialidase treated LMW-UK (20,000 IU) were applied to a column (110 ml). Each fraction contained 2.5 ml of the fractionated sample. The urokinase activity was determined by a fibrin plate method after each fraction was dialyzed overnight against 0.1 M phosphate buffer (pH 7.2). The most acidic peak at 280 nm was not caused by urokinase protein, but by degradation of Ampholine. ○, urokinase activity; □, absorbance at 280 nm; △, pH. a) HMW-UK, b) LMW-UK, c) sialidase treated LMW-UK.

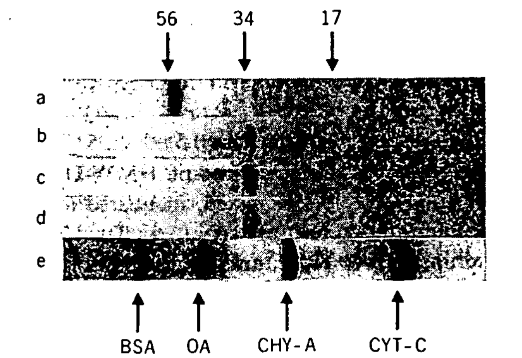

Fig. 2. SDS polyacrylamide gel electrophoresis of HMW-UK and LMW-UK. Reduction of the samples was performed with 1.5% dithiothreitol for 18 h at 20°C and the sample diluent consisted of 1% SDS in 0.01 M phosphate buffer (pH 7.2). The electrophoresis was carried out at 8 mA for 3.5 h, using 10 μg of a sample per tube. The numbers above gels show the molecular weights in thousands. a) HMW-UK, b) reduced HMW-UK, c) LMW-UK, d) reduced LMW-UK, e) standard protein.

TABLE I. The properties of HMW-UK and LMW-UK. The molecular weight of HMW-UK by SDS gel electrophoresis was estimated as the sum of the heavy (34,000) and the light (17,600) chains as shown in Fig. 2. The urokinase activity was determined by the Two Step method described in "EXPERIMENTAL PROCEDURE." a) and b) were determined using molecular weights of 51,600 for HMW-UK and 34,500 for LMW-UK. The theoretical specific activity was estimated by dividing the specific activity by % of active enzyme.

	HMW-UK	LMW-UK
Molecular weight		
Gel filtration	51,000	33,000
SDS gel electrophoresis	51,600	34,500
Specific activity (IU/mg)	157,400	246,700
IU/mol of urokinase[a]	8.34×10^{12}	9.68×10^{12}
% of active enzyme[b]	97.4	87.9
Theoretical specific activity (IU/mg)	161,600	280,700
Isoelectric point	9.70	6.85-9.37

that the half-cystine and aspartic acid contents of LMW-UK were lower than those of HMW-UK, which were in good agreement with the results of Soberano *et al.* (5).

Kinetic Studies of Urokinase—Table III indicates that the kinetic constants (K_m and k_{cat}) of HMW-UK toward two synthetic substrates (GGA-MCA and AGLMe) differed only slightly from those of LMW-UK. Therefore, their enzyme efficiency values (k_{cat}/K_m) were, in fact, identical.

TABLE II. Amino acid compositions and carbohydrate contents of HMW-UK and LMW-UK. The amino acid compositions were estimated as average or extrapolated values of 22–72 h hydrolysates.

	mol %		Residues per 51,600 mol. wt.[a]	Residues per 34,500 mol. wt.
	HMW-UK	LMW-UK	HMW-UK	LMW-UK
Tryptophan[b]	3.08	2.46	13.2	6.8
Lysine	6.74	7.14	28.9	19.7
Histidine	4.50	4.02	19.3	11.1
Arginine	5.30	5.58	22.7	15.4
Aspartic acid	8.80	6.85	37.7	18.9
Threonine[c]	6.28	7.14	26.9	19.7
Serine[c]	7.75	7.93	33.2	21.9
Glutamic acid	9.85	10.62	42.2	29.3
Proline	5.51	6.61	23.6	17.0
Glycine	9.50	9.06	40.7	25.0
Alanine	4.22	4.35	18.1	12.0
1/2 Cystine	4.76	2.97	20.4	8.2
Valine	4.32	4.20	18.5	11.6
Methionine	1.63	1.67	7.0	4.6
Isoleucine	4.18	5.80	17.9	16.0
Leucine	6.70	7.32	28.7	20.2
Tyrosine	4.25	4.17	18.2	11.5
Phenylalanine	2.66	2.72	11.4	7.5
Total no. of residues			428.6	276.4
Neutral sugar[d]	14.5	13.9		
Amino sugar[d]	19.0	25.0		
Sialic acid[d]	n.d.	n.d.		

[a] Molecular weight derived from the sum of the heavy (34,000) and the light (17,600) chains of HMW-UK as described in Table I. [b] The tryptophan content was determined by absorbance at 288 nm in 0.02 M phosphate buffer (pH 6.5)/6 M guanidine. [c] Value extrapolated to zero time. [d] Value in μg/mg protein. n.d.; not detected.

TABLE III. Enzyme kinetic studies of HMW-UK and LMW-UK. Active site titration of plasmin with *p*-nitrophenyl-*p'*-guanidinobenzoate showed that one CU of plasmin corresponded to 15.6 nmol of plasmin. k_{cat}, catalytic rate constant toward a synthetic substrate; k_{plg}, catalytic rate constant toward Glu-plasminogen; K_m, dissociation constant for a synthetic substrate; K_{plg}, dissociation constant for Glu-plasminogen.

	HMW-UK			LMW-UK		
	k_{cat} (k_{plg}) (s^{-1})	K_m (K_{plg}) (M)	k_{cat}/K_m (k_{plg}/K_{plg}) ($M^{-1}\cdot s^{-1}$)	k_{cat} (k_{plg}) (s^{-1})	K_m (K_{plg}) (M)	k_{cat}/K_m (k_{plg}/K_{plg}) ($M^{-1}\cdot s^{-1}$)
AGLMe	384.6	2.5×10^{-3}	1.54×10^{5}	428.8	3.3×10^{-3}	1.30×10^{5}
GGA-MCA	9.9	4.4×10^{-4}	2.27×10^{4}	10.2	3.9×10^{-4}	2.66×10^{4}
Plg-fibrin	0.74	1.3×10^{-6}	5.59×10^{5}	0.50	3.1×10^{-6}	1.63×10^{5}
Plg-S2251	0.98	2.3×10^{-6}	4.29×10^{5}	1.31	5.9×10^{-6}	2.24×10^{5}

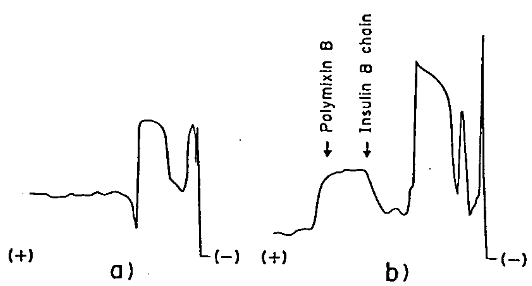

Fig. 3. SDS polyacrylamide gel electrophoresis of LMW-UK and reduced LMW-UK. Reduction of the samples (400 μg per tube) was performed as described in Fig. 2. The acrylamide concentration was 15%. The arrows indicate the positions of polymixin B (1,200) and the B-chain of insulin (3,400). After gels were stained, they were scanned with a densitometer at 500 nm. a) unreduced LMW-UK, b) reduced LMW-UK.

On the other hand, when the Glu-plasminogen-fibrin coupled assay system was employed, it was found that the dissociation constant for activation of plasminogen (K_{plg}) was 2.4 fold lower than LMW-UK, and the enzyme efficiency value (k_{plg}/K_{plg}) of HMW-UK was found to be 1.9 fold higher than that of LMW-UK. This was also found with the Glu-plasminogen-VLL-pNA coupled assay system. It was revealed in this study that K_{plg} were 100–1,000 fold lower than K_m.

Autocatalytic Conversion of HMW-UK to LMW-UK—Figure 4 shows the time dependent change of the SDS gel electrophoretic pattern of HMW-UK during incubation at 37°C for 72 h at pH 7.2. 34,000- and 17,000-dalton bands newly appeared after 5 h incubation and their intensities increased in a time dependent manner, in contrast to the decrease in the intensity of the 56,000-dalton band. At 0 h only the 56,000-dalton band showed significant urokinase activity. After 72 h the activity, however, was detectable in not only the 56,000-dalton band but also the 34,000-dalton band, while the 17,000-dalton band showed no activity. Benzamidine, with which urokinase was incubated, apparently inhibited the change of the SDS gel electrophoretic pattern, in contrast to aprotinin which did not suppress the change. The urokinase activity was 338,400 IU/ml before incubation and 342,400 IU/ml after incubation. It was also revealed that the HMW-UK preparation used here contained no detectable caseinolytic activity.

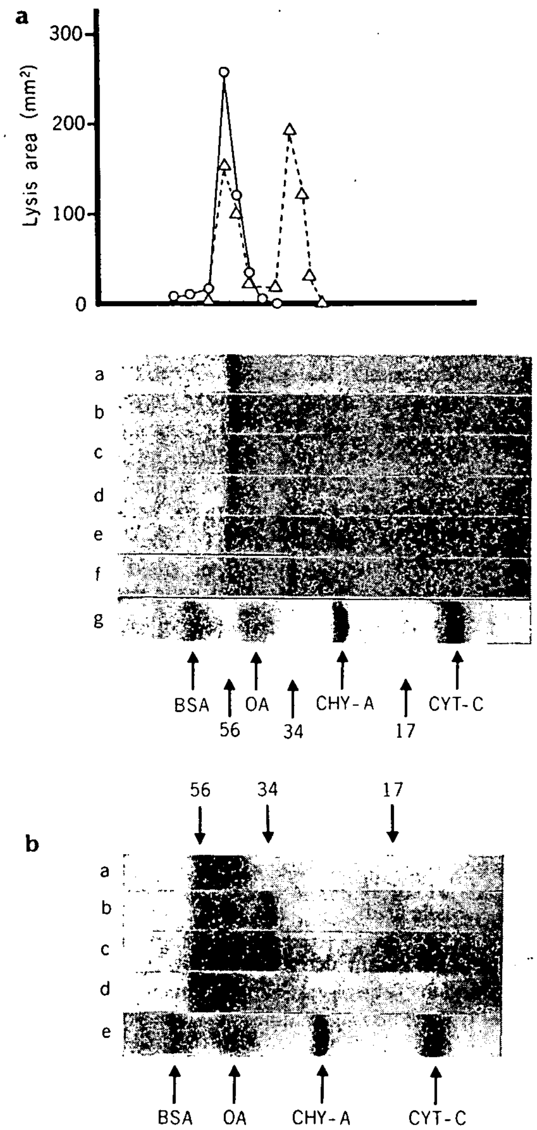

Fig. 4. The autocatalytic conversion of HMW-UK to LMW-UK. Figure 4-a shows the change of the electrophoretic pattern of HMW-UK incubated alone. a) 0 h, b) 5 h, c) 17 h, d) 24 h, e) 48 h, f) 72 h, g) standard protein, ○ urokinase activity at 0 h incubation, △ urokinase activity at 72 h incubation. Figure 4-b shows the change of HMW-UK either in the presence of benzamidine or aprotinin as described in "EXPERIMENTAL PROCEDURE." a) 0 h, control, b) 72 h, control, c) 72 h with 500 KIU/ml of aprotinin, d) 72 h with 5 μM of benzamidine, e) standard protein. All samples were incubated at 37°C and pH 7.2. The numbers beneath (Fig. 4-a) or above (Fig. 4-b) gels show the molecular weights in thousands.

DISCUSSION

The purpose of this study was to clarify 1) whether LMW-UK is composed of two chains the same as HMW-UK is, 2) whether HMW-UK is autocatalytically converted into LMW-UK and 3) the differences in their physicochemical and enzymological properties.

Amino acid analysis showed 1.7–1.9 fold numbers of acidic amino acids, compared with those of basic amino acids in both urokinases. Thus, the basic isoelectric point of HMW-UK (pH 9.7) appears to be due to the presence of the amide forms of acidic amino acids since a very small amount of amino sugar probably does not contribute to the high p*I*. The result that LMW-UK has multiple isoelectric subforms is in excellent agreement with those of Soberano *et al.* (*5*) and Ogawa *et al.* (*6*). It is, however, uncertain why LMW-UK has multiple p*I*s, since it might be derived from HMW-UK. It can be ruled out that the multiple subforms are due to the differences in quantities of sialic acid and amino sugar, as we have indeed observed that 1) the p*I*s are not influenced by treatment with sialidase, and sialic acid is undetectable, and 2) only a very small amount of amino sugar is present. Thus, it can be presumed that there are some differences in the quantities of amide forms of acidic amino acids among the five subforms of LMW-UK.

Kinetic studies of urokinase toward two synthetic substrates and Glu-plasminogen, *i.e.* a natural substrate of urokinase, revealed that HMW-UK gives the same kinetic constants as LMW-UK toward the synthetic substrates, but HMW-UK has a higher affinity than LMW-UK toward the natural substrate. Therefore, HMW-UK activates more effectively Glu-plasminogen to plasmin.

It became apparent that HMW-UK is composed of heavy (34,000) and light (17,600) chains in agreement with Soberano *et al.* (*5*). Although it has not been reported yet that LMW-UK also has two chains, a small peptide with a molecular weight of 1,200–3,400 was clearly detected on SDS gel electrophoresis in the presence of dithiothreitol by us (Fig. 3).

We have further observed that HMW-UK is converted into LMW-UK in an autocatalytic conversion manner, which is supported by the following results; 1) the conversion was inhibited by benzamidine, a potent inhibitor of urokinase, but not by approtinin, 2) the sample contained no detectable protease activity which will cause the conversion, and 3) the newly appearing 34,000-dalton protein after 72 h incubation, which has in fact a molecular weight identical to LMW-UK, had urokinase activity.

Thus, it can be concluded that LMW-UK, derived from HMW-UK, is also composed of two chains linked by disulfide bond(s).

During the autocatalytic conversion of HMW-UK, a protein band slightly lower than HMW-UK (Fig. 4) was detected in the gels. It is deduced that this protein is an intermediate form of urokinase although the urokinase activity of this protein has not been measured. Endo-group analysis of the two chains of both forms of urokinase will make our conclusion clear, but we would like to mention now that not only HMW-UK but also LMW-UK has two chains.

We conclude here that HMW-UK with a higher affinity toward Glu-plasminogen is converted into LMW-UK with a lower affinity in an autocatalytic conversion manner.

REFERENCES

1. Astrup, T. & Sterndoroff, I. (1953) *Proc. Soc. Exp. Biol. Med.* **81**, 675–678
2. Sobel, G.W., Mohler, S.R., Jones, N.W., Dowdy, A.B.C., & Guest, M.M. (1952) *Am. J. Physiol.* **171**, 768–769
3. Lesuk, A., Terminiello, L., & Traver, J.H. (1965) *Science* **147**, 880–882
4. White, W.F., Barlow, G.H., & Mozen, M.M. (1966) *Biochemistry* **5**, 2160–2169
5. Soberano, M.E., Ong, E.B., Johnson, A.J., Levy, M., & Schoellmann, G. (1976) *Biochim. Biophys. Acta* **445**, 763–773
6. Ogawa, N., Yamamoto, H., Katamine, T., & Tajima, H. (1975) *Thromb. Diath. Heamorrh.* **34**, 194–209
7. Holmberg, L., Bladh, B., & Asted, B. (1976) *Biochim. Biophys. Acta* **445**, 215–222
8. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
9. Spackman, R.H., Stein, W.H., & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
10. Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
11. Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
12. Elson, L.A. & Morgan, W.T.J. (1933) *Biochem. J.* **27**, 1824–1828

13. Anno, K. & Seno, N. (1968) *Protein, Nucleic acid and Enzyme* (in Japanese) Suppl. 15–26
14. Plough, J. & Kjeldgaard, N.O. (1957) *Biochim. Biophys. Acta* **24**, 278–282
15. Nishizaki, S. & Kawamura, J. (1974) *Iyakuhin Kenkyu* (in Japanese) **5**, 295–308
16. Chase, T., Jr. & Shaw, E. (1969) *Biochemistry* **8**, 2212–2224
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265–275
18. Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T., & Sakakibara, S. (1977) *J. Biochem.* **82**, 1495–1498
19. Johnson, A.J., Kline, D.L., & Alkjersig, N. (1969) *Thromb. Diath. Heamorrh.* **21**, 259–272
20. Friberger, P., Knös, M., Gustavsson, S., Aurell, L., & Cleason, G. (1978) *Haemostasis* **7**, 138–145

Studies on the Ca^{2}
Rabbit Skeletal Mu
the 80 K and the 3

Shuichi TSUJI

Department of B
of Tokyo, Hongo

Received for pub

The structure
skeletal muscle
disc gel electrop
(30 K)) on SD
was separated a
unit. The resu
activity is prese
30 K subunit is

Recently we purified Ca
teinases (CANPs) of skel
sources (*1*, *2*). These C
various properties except
The chicken proteinase is
weight 80,000 (*1*). On the
from mammalian source
(this paper), are compos
molecular weights of 8
30,000 (named 30 K). T
lian muscle CANP are c

Abbreviations: CANP, C
teinase; SDS, sodium do
dithiobis(2-nitrobenzoic aci
of Ca^{++} ions which gives h
80 K and 30 K proteins, t
with molecular weights of
tively; Buffer A, 20 mM T
EDTA, 10 mM 2-mercapto
same as buffer A but pH
HCl, 0.1 mM EDTA, 1 mM